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IDENTIFICATION OF MULTIPLE PATHOGENIC BACTERIA USING A DNA MICROARRAY

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The primary technique currently used to detect biological agents is based on immunoassays. Although sensitive and specific, immunoassays may not produce accurate identification based on single target detection and cannot determine subtle differences in the genome. Gene arrays can hybridize multiple DNA targets simultaneously, and thus, have enormous potential for detection and identification of pathogens. In this study, pathogenic *E. coli* O157:H7-specific genes, nonpathogenic K12-specific genes, common *E. coli* genes, and negative control genes were PCR-amplified and printed onto the surface of glass slides. Further, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Neisseria meningitides* specific genes were also printed. After labeled bacterial cDNA samples were hybridized with probes on the microarray, specific fluorescence patterns were obtained, enabling identification of pathogenic *E. coli* O157:H7, nonpathogenic *E. coli* K12, antibiotic-resistant strain, and three other pathogenic bacteria. Because multiple datapoints are accessed, we demonstrate that this array method is more efficient and accurate than a typical immunoassay, which detects a specific protein product.

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PREFACE

The work described in this report was authorized under Project No. CPD2016, Strain-Specific Pathogen Identification Using Total DNA Amplification and Gene Chip Based Detection. The work was started in May 2001 and completed in April 2002.

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1. INTRODUCTION

The enterohemorrhagic *Escherichia coli* (EHEC), such as strain O157:H7, causes capillary thrombosis, diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) by producing Shiga-like toxins (Ryan et al., 1994). Large outbreaks of O157:H7 infection have occurred all over the world, including Fukuoka, Okayama, Osaka, and Hiroshima, Japan, in 1996 (Izumiya et al., 1997), central Scotland in 1996 (Dundas et al., 2001), Germany from 1988 through 1998 (Liesegang et al., 2000), and multistate outbreaks in the United States in the past few years (Jaeger and Acheson, 2000; Mohle-Boetani et al., 2001). A reliable and accurate detection method could help to prevent, diagnose, and treat the pathogens. Typically, the presumptive identification of O157:H7 in the clinical laboratory is done by screening bacteria on Sorbitol-MacConkey (SMAC) agar because O157:H7 do not ferment D-sorbitol well and appear as colorless colonies in an otherwise pink population of sorbitol-fermenting organisms (March and Ratnam, 1986). To prevent false identification, a confirmative test is performed by immunoassay against either the O157:H7 flagellar antigen (He et al., 1996; Seah and Kwang, 2000) or the Shiga-like toxins (Ludwig et al., 2001). However, O157 family members may be either H7 or non-H7 and may have only Shiga-like toxin 1, only Shiga-like toxin 2, or both. It was even reported that some O157 strains without Shiga-like toxin genes (*stx*) could still cause severe diarrhea and hemorrhagic colitis (Allerberger et al., 2000; Feng et al., 2001). A fluorescent 4-methylumbelliferyl-β-D-glucuronide cleavage assay has been used to detect the activity of β-glucuronidase, which does not appear in most O157 (Thompson et al., 1990). However, a particular O157 (G5101) isolated from a patient with bloody diarrhea was β-glucuronidase positive (Hayes et al., 1995). The pulsed-field gel electrophoresis (PFGE) method was based on comparing the endonuclease digestion patterns to identify O157:H7 (Izumiya et al., 1997). But, in some experiments using this method, the *stx*-negative strains were not distinguishable from *stx*-positive strains (Allerberger et al., 2000). Alternatively, an oligonucleotide probe, PF-27, has been designed to detect a conserved mutation in the *uid*A gene of O157:H7 (Feng, 1993). Since detecting solely one gene or one specific enzyme product does not produce reliable identification, a combination of the above methods is commonly performed.

The complete genome of O157:H7 EDL933 was sequenced and recently published (Perna et al., 2001). The O157:H7 EDL933 genome (5,528,970 bp) is significantly larger than the K12 MG1655 genome (4,639,221 bp) that was also sequenced previously (Blattner et al., 1997). By comparing the genes of O157 with those of the nonpathogenic strain K12 MG1655, strain-specific genes were identified. The investigators selected multiple strain-specific genes as well as many common genes and used them as probes to screen genotypes of given bacteria through simultaneous hybridization, enabling more reliable and accurate identification. To accomplish this approach, the DNA microarray technology was employed. This technology has been commonly used to analyze transcription levels of genes under different environmental conditions (DeLisa et al., 2001; Robles et al., 2001; Varedi et al., 2001), in different diseases (Hata et al., 2001; Sorlie et al., 2001), in the presence of various toxicants

(Gerhold et al., 2001), and in the presence of various pharmacological substances (Marcotte et al., 2001). The ability of the gene array to simultaneously hybridize a low quantity of nucleotides with multiple targets provides a very efficient way of confirming genotypes. Finally, three medically important pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Neisseria meningitides*) DNA were also printed to test whether one array could discriminate multiple bacteria. *S. aureus* produces enterotoxin, exotoxin, leukotoxin and toxic shock syndrome toxin, and is the major cause of nosocomial infection and food poisoning (McGahee and Lowy, 2000; Dinges et al., 2000). *S. pneumoniae* is the major cause of pneumonia, meningitis, and otitis media. Its virulence comes from the capsular polysaccharide coat and pneumolysin (Garcia et al., 1999; Mitchell, 2000). Untreated *N. meningitides* infection results in septicemia and meningitis. The virulence is based on its anti-phagocytic polysaccharide capsule (Tzeng and Stephens, 2000; Brandtzaeg et al., 2001).

2. MATERIAL AND METHODS

2.1 Strains and DNA Sources.

The *E. coli* O157:H7 EDL933, K12 MG1655 and JM107 were obtained from the American Type Culture Collection (ATCC). The strain K12 ER2267 was obtained from New England Biolabs, Incorporated (Beverly, MA). Another strain, BL21, carrying a plasmid, pOPH (with ampicillin resistance), was described previously (Wu et al., 2001). The genomic DNAs of these strains were isolated using the Easy-DNA kit (Invitrogen). Genomic DNAs of *Staphylococcus aureus*, *Streptococcus pneumoniae,* and *Neisseria meningitides* were obtained from ATCC. Control DNA fragments such as *EGFR*, *GAPDH*, and antibiotics resistance genes (amp^R and tet^R) were obtained from vectors, pTRI [Ambion, Incorporated (Austin, TX)] and pBR322 [Invitrogen Life Technologies (Carlsbad, CA)].

2.2 Probe Selection and Amplification.

Complete comparison of genomic DNA between O157:H7 EDL933, a pathogenic strain, and K12 MG 1655, a common nonpathogenic strain, was based on the data published recently (Blattner et al., 1997; Perna et al., 2001). Genomic sequences of *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Neisseria meningitides* were also published recently (Kuroda et al., 2001; Tettelin et al., 2001; Tettelin et al., 2000, respectively). The strain-specific sequences were carefully selected according to the low similarity between each other and other species, as well as their size (either very short or long genes were not easily amplified, recovered, and/or hybridized). Thus, the genes were chosen after running the web-based Blast program [National Center for Biotechnology Information (Bethesda, MD)]. All, polymerase chain reaction (PCR) primers were designed using the freely available software, Primer3 (`http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/`). The oligomers were purchased from Ransom Hill Bioscience, Incorporated (Ramona, CA). The genes were amplified by PCR using either Platinum PCR supermix [Invitrogen Life Technologies (Carlsbad, CA)] or Vent polymerase (NEB) with each of the genomes listed above as templates. After PCR, to verify the size and quantity of the PCR products, the genes were purified and checked on agarose gels.

2.3 Microarray Printing and Processing.

The CMT-GAPS II slides [Corning, (Corning, NY)] were used as substrates for the arrays. The samples were loaded in 96-well plates (Corning) and were printed on coated slides using Affymetrix 417 arrayer [Affymetrix, Incorporated (Santa Clara, CA)]. The distance between each spot was 500 μm, and each gene was spotted in triplicate. The temperature was 22-23 °C, and the humidity was 70% during the printing process. The DNA was immobilized on the surface by baking the slides at 80 °C for 3 hr.

2.4 Genomic DNA Labeling.

Instead of labeling RNA of *E. coli*, the most commonly employed protocol, we labeled the *E. coli* genomic DNA directly using the Klenow fragment enzyme. The O157:H7 EDL933 DNA was labeled with Cy5-dCTP and K-12 ER2267 DNA was labeled with Cy3-dCTP. The labeling preparations were incubated at 37 °C for 2 hr and then purified with Microcon YM-30 filters [Millipore (Bedford, MA)]. The final volume of mixed DNA solution was concentrated to <10 μL and stored in the dark at 4 °C until hybridization. In the comparison experiments, the DNA from BL21 carrying pOPH was labeled with Cy3-dCTP, and the DNA from JM107 was labeled with Cy5-dCTP with the same protocol. *S. aureus, S. pneumoniae*, and *N. meningitides* DNAs were labeled with Cy5-dCTP, Cy3-dCTP, and Cy5-dCTP, respectively.

2.5 Hybridization.

The sample in hybridization buffer was applied to the spotted area on a pre-hybridized slide. The microarray was covered by Hybri-Slip cover slip [Sigma (St. Louis, MO)] and placed in the hybridization chamber overnight at 42 °C. When the hybridization was finished, the microarray was washed in the following successive steps: (1) 2X SSC, (2) 0.1X SSC plus 0.1% SDS, and (3) 0.1X SSC.

2.6 Scanning and Analysis.

The hybridized slide was scanned using an Affymetrix 428 scanner [Affymetrix, Incorporated (Santa Clara, CA)]. False color images of Cy5 and Cy3 signals were produced with the free software, Scanalyze, obtained from the Eisen Laboratory [Lawrence Berkeley National Laboratory (Berkeley, CA)] http://rana.lbl.gov/EisenSoftware.htm.

3. RESULTS AND DISCUSSION

The table lists the genes selected for this experiment. Spots A1-A12, B1-B12, and C1 contained O-157:H7 specific genes. Spots C2-C12 contained K12-specific genes. Spots D1-D12, E1, and E2 contained common *E. coli* genes. Many of these common genes such as *mcr*ABC, *hsd*RS, *omp*T, *rec*A, and *pro*AB were either selectively mutated or deleted in different strains. By using these probes, the detailed identification of *E. coli* strains was made possible. Spots E3-E6 contained control genes that do not appear in *E. coli*. Detection of B9 (*stx*1) and B10 (*stx*2) was critical in identifying pathogenic O157:H7 because these genes encode shiga-like

toxin subunits (AE005442 and AE005296). B11 (*eae*) is also an important marker for O157 because it encodes the intimin adherence protein that helpes bacteria attach to intestinal cells (AE005595). The gene spotted at C1 (*tir*) produces a translocated intimin receptor, which is a characteristic for enteropathogenic and enterohaemorrhagic *E. coli* (Frankel et al., 2001). The functions of many genes are still unknown. However, based on the Blast comparison, these genes were thought to be good strain-specific markers. Probes from pathogenic *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Neisseria meningitides* were arranged in plate 2.

Table. Genes Printed on the Microarray

PLATE 1

O157 EDL933-specific genes

A1	*intH*	integrase
A2	*z1019*	secreted effector
A3	*z0894*	glutamate mutase
A4	*sepQ*	transportation
A5	*z0020*	unknown
A6	*z1866*	integrase
A7	*z2323*	unknown
A8	*z4183*	unknown
A9	*z3617*	unknown
A10	*z3206*	degradation
A11	*z3161*	unknown
A12	*z4326*	enterotoxin
B1	*z5212*	unknown
B2	*z4881*	adolase
B3	*z4810*	unknown
B4	*z5686*	kinase
B5	*z5692*	kinase
B6	*z5429*	unknown
B7	*z5337*	unknown
B8	*z5878*	integrase
B9	*stx1BA*	toxin
B10	*stx2AB*	toxin
B11	*eae*	intimin adherence protein
B12	*escD*	peptide secretion
C1	*tir*	translocated receptor

K12 MG1655-specific genes

C2	*yi81_1*	transposon-related
C3	*intD*	integrase
C4	*trs5_3*	transposase
C5	*feaR*	regulatory protein for the 2-phenylethylamine catabolism
C6	*feaB*	phenylacetaldehyde dehydrogenase
C7	*rspA*	starvation sensing protein
C8	*rspB*	starvation sensing protein
C9	*b2442*	integrase
C10	*xapB*	xanthosine permease
C11	*atoS*	sensor protein for degradation regulator atoC
C12	*lyxK*	L-xylulose kinase

Table. Genes Printed on the Microarray (Continued)

Common *E. coli* genes

D1	*mcrA*	degradation of DNA
D2	*mcrB*	degradation of DNA
D3	*mcrC*	degradation of DNA
D4	*ompT*	protease
D5	*lon*	protease
D6	*lacI*	transcriptional repressor
D7	*hsdR*	DNA restriction
D8	*hsdS*	DNA restriction
D9	*endA*	endonuclease
D10	*recA*	recombination
D11	*lacZ*	beta-D-galactosidase
D12	*proA*	glutamylphosphate reductase
E1	*proB*	glutamate kinase
E2	*luxS*	AI-2 synthase

Control genes

E3	*ampR*	ampicillin resistance
E4	*tetR*	tetracycline resistance
E5	*GAPDH*	dehydrogenase
E6	*EGFR*	growth factor receptor

PLATE 2

Staphylococcus aureus genes

A1	coa	staphylocoagulase precursor
A2	set8	exotoxin 8
A3	set9	exotoxin 9
A4	set10	exotoxin 10
A5	set11	exotoxin 11
A6	set12	exotoxin 12
A7	set13	exotoxin 13
A8	set14	exotoxin 14
A9	lukD	leukotoxin
A10	lukE	leukotoxin

Streptococcus pneumoniae genes

B1	sp0071	immunoglobulin A1 protease
B2	cps4a	capsular polysaccharide biosynthesis protein
B3	cps4b	capsular polysaccharide biosynthesis protein
B4	cps4d	capsular polysaccharide biosynthesis protein
B5	sp0966	adherence and virulence protein A
B6	sp1272	polysaccharide biosynthesis protein
B7	sp1529	polysaccharide biosynthesis protein
B8	sp1923	pneumolysin
B9	sp1937	autolysin

Table. Genes Printed on the Microarray (Continued)

Neisseria meningitides genes		
C1	siaC	polysialic acid capsule biosynthesis protein
C2	siaB	polysialic acid capsule biosynthesis protein
C3	synX	polysialic acid capsule biosynthesis protein
C4	ctrA	capsule polysaccharide export outer membrane protein
C5	ctrB	capsule polysaccharide export outer membrane protein
C6	ctrC	capsule polysaccharide export outer membrane protein
C7	ctrD	capsule polysaccharide export ATP-binding protein
C8	lipA	capsule polysaccharide modification protein
C9	lipB	capsule polysaccharide modification protein
C10	MviN	virulence factor MviN
C11	vapA	virulence-associated protein

Figure 1 illustrates the hybridization patterns of O157:H7 EDL933 and K12 ER2267. As seen in Figure 1 (a), significant signals appeared in spots A1-A12, C1, C2, E1-E2, B1-B12, D4, D5, D6, D9, D10, D11, and D12. Due to the good labeling efficiency and correct hybridization of the O157:H7-specific spots (A1-A12, B1-B12, and C1), the genes comprising these spots proved to be excellent probes for detecting O157:H7 EDL933. No K12-specific gene probes (C3-C12) were cross-reactive with the O157:H7 DNA except C2 (*yi81_1*), which expresses a hypothetical protein with transposon-related functions (AE000112). This is most likely due to an unusually high nonspecific binding of this gene. The common genes at spots D5 (*lon*), D9 (*end*A), D11 (*lacZ*), D4 (*omp*T), and D6 (*lac*I) demonstrated that each *E. coli* species was properly detected. Finally, the genes that do not exist in O157:H7 EDL933 were examined. Indeed, no signals from D1 (*mcr*A), D2 (*mcr*B), D3 (*mcr*C), D7 (*hsd*R), D8 (*hsd*S), E3 (*amp*R), E4 (*tet*R), E5 (*GAPDH*), and E6 (*EGFR*) were detected when assayed by O157:H7 EDL933 DNA. Overall, the hybridization results demonstrated that the microarray accurately detects the "fingerprint" of O157:H7 EDL933.

Figure 1 (b) illustrates the pattern for the nonpathogenic K12 ER2267. Clearly, the locations of hybridization signals are different from those of the O157:H7 strain. None of the O157:H7-specifc dots had significant signals. In contrast, almost all of the K12-specific genes in the second row of the image (C2-C12) produced strong signals [except C9 (*b2442*)], indicating correct identification of K12 sample DNA. Although b2442 exists in K12 MG1655, we suspect that it is missing in K12 ER2267 because of the minimal signal. The strong hybridization of these K12-specific spots demonstrates that these genes are excellent probes in distinguishing O157:H7 from K12. In the case of common gene group, both positive and negative hybridizations were observed. D1 (*mcr*A) is known to be absent in K12 ER2267 (*New England Biolabs catalog and technical reference*), and our results were confirmatory. Also, K12 ER2267 has large deletions on *mcr*BC (D2 and D3), as well as on *hsd*RMS restriction systems (D7 and D8) (*New England Biolabs catalog and technical reference*). Thus, it was reasonable to see null signals on these spots. The proteases, *lon* (D5) and *omp*T (D4) were present in K12 and were easily detected. In addition, the K12 ER2267 strain, reported to be positive for *lac*I, *pro*A, and

*pro*B (*New England Biolabs catalog and technical reference*), is confirmed by signals on these spots [D6 (*lacI*), D12(*proA*), and E1(*proB*)]. Although the activities of *end*A (D9), *rec*A (D10), and *lacZ* (D11) were reportedly abolished (*New England Biolabs catalog and technical reference*), these genes expressed strong signals on the array. A reasonable assumption is that these sequences were mutated by a frame shift, point mutation, or a similar method instead of a large deletion. Thus, these genes could still be detected on the microarray. An analogy was the case that O157:H7 had negative β-glucuronidase activity, but it did possess a mutated form of this gene (*uid*A) with several base mutations (Feng, 1993). Finally, as expected, negative control spots [E3 (*amp*R), E4 (*tet*R), E5 (*GAPDH*), and E6 (*EGFR*)] did not produce any signals.

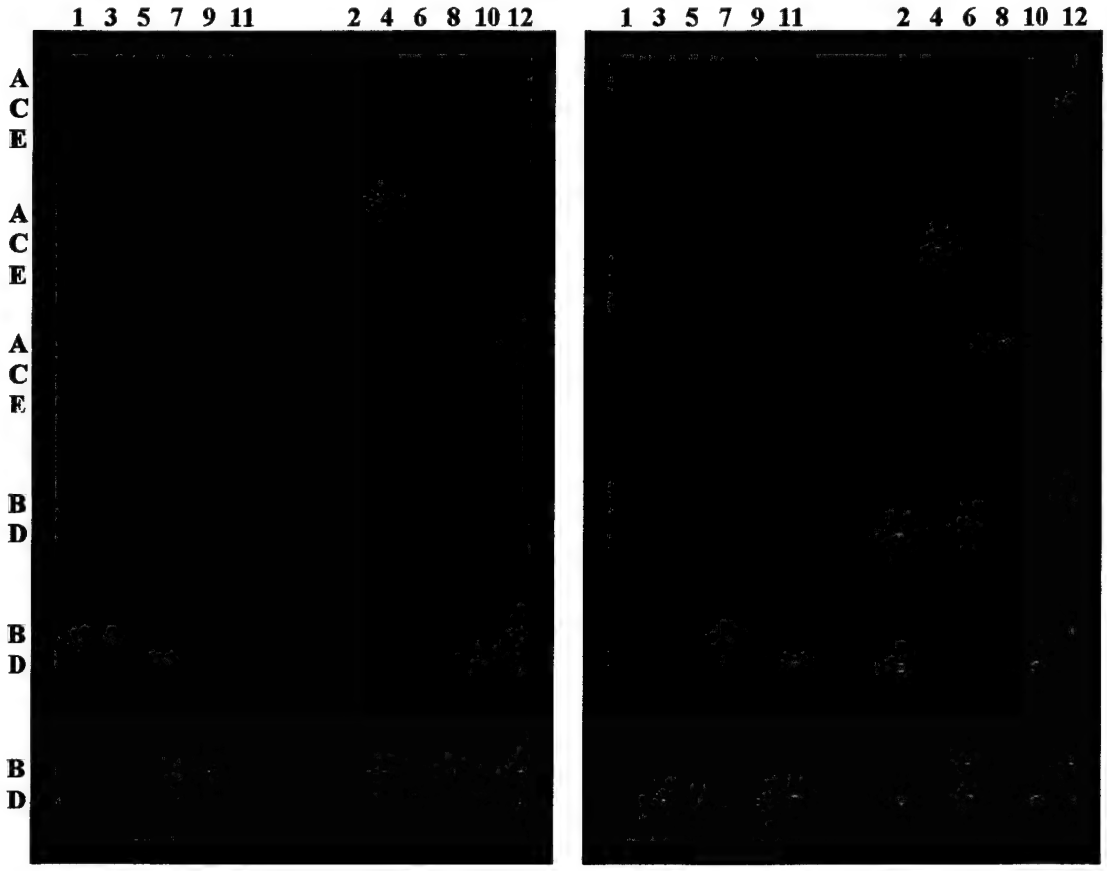

Figure 1. Hybridization Patterns of (a) Cy5-labeled O157:H7 EDL933 Genomic DNA and (b) Cy3-labeled K12 ER2267 Genomic DNA

Overall, at least 90% of the selected genes in our microarray solidly confirmed the genomes of our tested O157:H7 EDL933 and K12 ER2267 strains. By comparing the hybridization patterns, the pathogenic and nonpathogenic *E. coli* could be readily distinguished.

A complete overlapping image with both O157:H7 EDL933 and K12 ER2267 signals is shown in Figure 2.

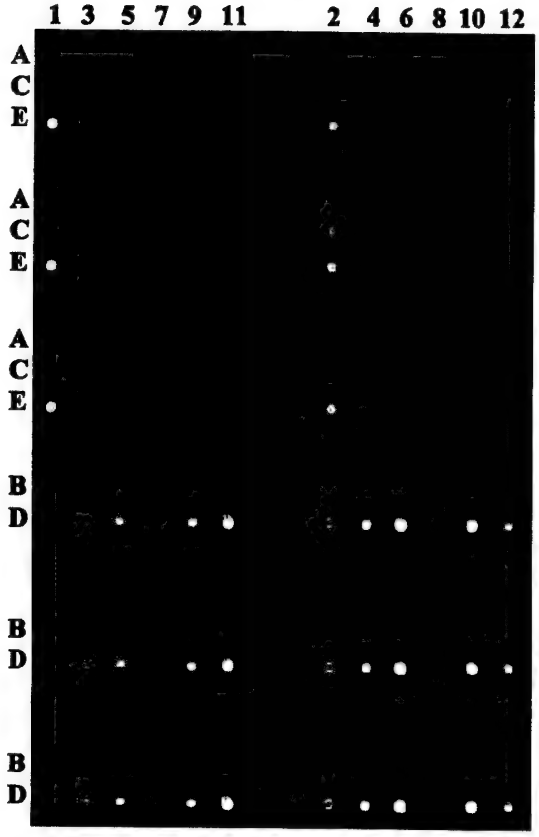

Figure 2. Microarray Image Generated by Overlapping Both O157:H7 EDL933 and K12 ER2267 Signals

In this figure, red spots confirm that all O157-specific genes are printed in the first rows in all four quadrangles as well as in the C1 position. The green spots confirm that all K12-specific genes are printed from C2-C12. Exceptions include C2, which was somewhat yellow due to nonspecific binding of O157:H7 DNA, and C9 (*b2442*), which was presumed absent in K12 ER2267. Common *E. coli* genes, E1(*pro*B), E2(*lux*S), D4(*omp*T), D5(*lon*), D6(*lac*I), D9(*end*A), D10(*rec*A), D11(*lac*Z), and D12(*pro*A) are seen as yellow signals among all others. This color confirmed their appearance in both O157 and K12. Dark spots at D1(*mcr*A), D2(*mcr*B), D3(*mcr*C), D7(*hsd*R), and D8(*hsd*S) confirm that these genes are absent in both strains. Spots E3-E6 were also dark, demonstrating correct lack of hybridization at these negative control spots. The noise from nonspecific binding shown in Figure 1 was significantly reduced after overlapping the two images. Although slight cross reactivity was still observed (e.g., D2 and D3), it was relatively weak compared to the true hybridization in other spots. Overlapping two images generates a more definite result since the noise is normalized twice (by each color).

Most laboratory *E. coli* strains are derivatives of the K12 or B strains (*New England Biolabs catalog and technical reference*). Thus, we also tested *E. coli* BL21 (B strain derivative) and JM107 (K12 derivative) to investigate whether their patterns were still

distinguishable from the pathogenic O157:H7. We further transformed the BL21 with an ampicillin resistant plasmid to test whether the microarray could correctly detect a genetically engineered drug-resistant strain. The results are shown in Figure 3. Figure 3(a) shows that non-pathogenic BL21 possessed K12-specific genes (C2-C12 except *b2442*) but not O157 specific genes (row A, row B, and C1). An exception was A8 (*z4183*), which showed some signal. BL21 is an *ompT*- strain, and the lack of a signal at spot D4 (*ompT*) confirmed this fact. Importantly, spot E3 (amp^R) correctly detected the ampicillin resistance gene on the plasmid. As expected, JM107 [Figure 3(b)], showed that 10 out of 11 K12-specific genes were conserved in the strain (except *b2442*), and no O157:H7 specific spots produced signals except A8 (*z4183*). We are not sure whether the hybridization of A8 (*z4183*) was due to nonspecific binding. However, more than 30 definite results in rows A, B, and C clearly identified the nonpathogenic strain. Lastly, JM107 is known to be *proA+ proB+ mcrA- lacI*^{q} (ATCC), and the hybrizidation results at D12, E1, D1, and D6 confirmed this genotype. However, two curious negative results are noted here: First, *E. coli* B strain should be devoid of Lon activity (*New England Biolabs catalog and technical reference*). However, D5 (*lon*) in Figure 3(a) produced signal. Second, the *lacZ* mutation in JM107 was known to be a large deletion but there was still signal at D11 (*lacZ*) in Figure 3(b). We hypothesize that if these genes were not completely absent (i.e., partial deletion), one might still detect hybridization. Nevertheless, correct signals from more than 90% of the tested genes provided solid identification.

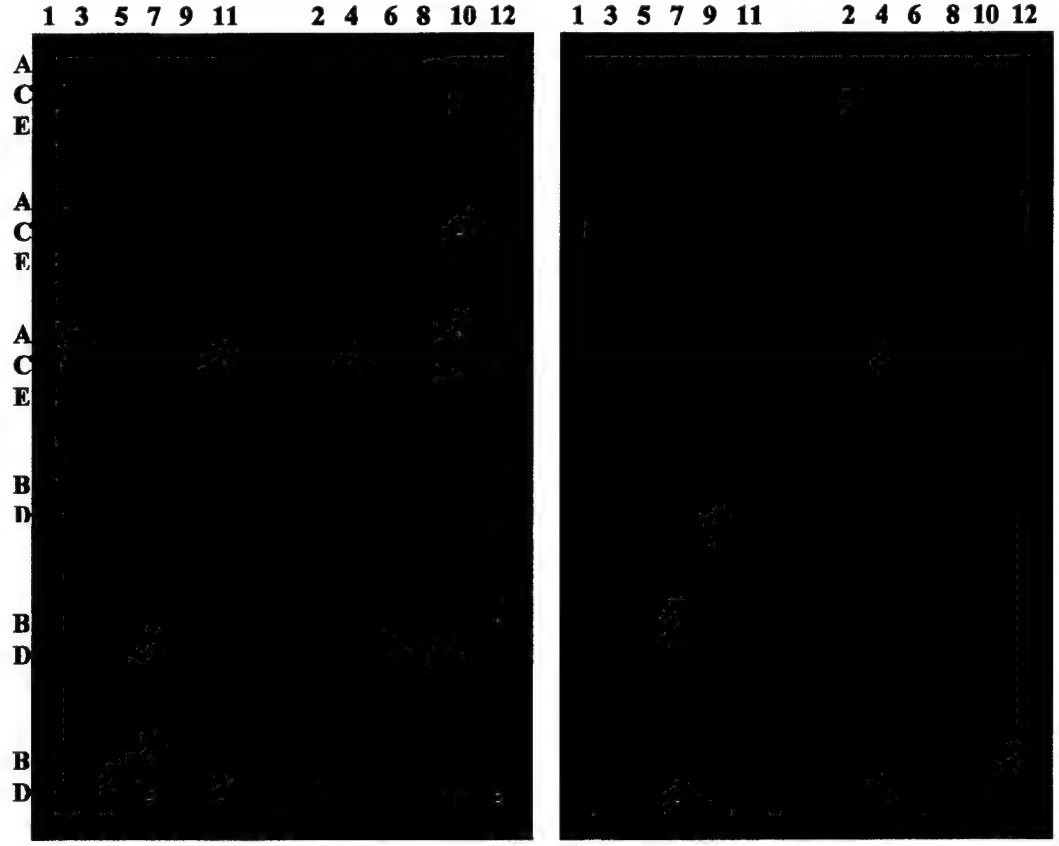

Figure 3. Hybridization Patterns of (a) *E. coli* BL21 Genomic DNA Labeled with Cy3-dCTP, and (b) *E. coli* JM107 Genomic DNA Labeled with Cy5-dCTP

Figure 4 demonstrates that the microarray can identify four different pathogenic bacteria without cross-reactivity. The O157:H7 DNA sample showed signals on the upper left panel only. As expected, *N. meningitidis* showed signals in row C on the upper right panel. *S. pneumoniae* probes were arranged in row B in plate 2, and signals in the lower right panel confirmed the identity of DNA sample. When the *S. aureus* sample was hybridized on the array, only row A on the upper right panel showed signals. In all cases, no nonspecific binding between different bacterial DNA was observed, demonstrating that these probes were excellent in identifying these four bacteria. Again, since multiple probes and spots in triplicate were employed, a solid confirmation of bacteria was achieved. The signals on the array were very clear with minimal background, indicating that our protocol was successful.

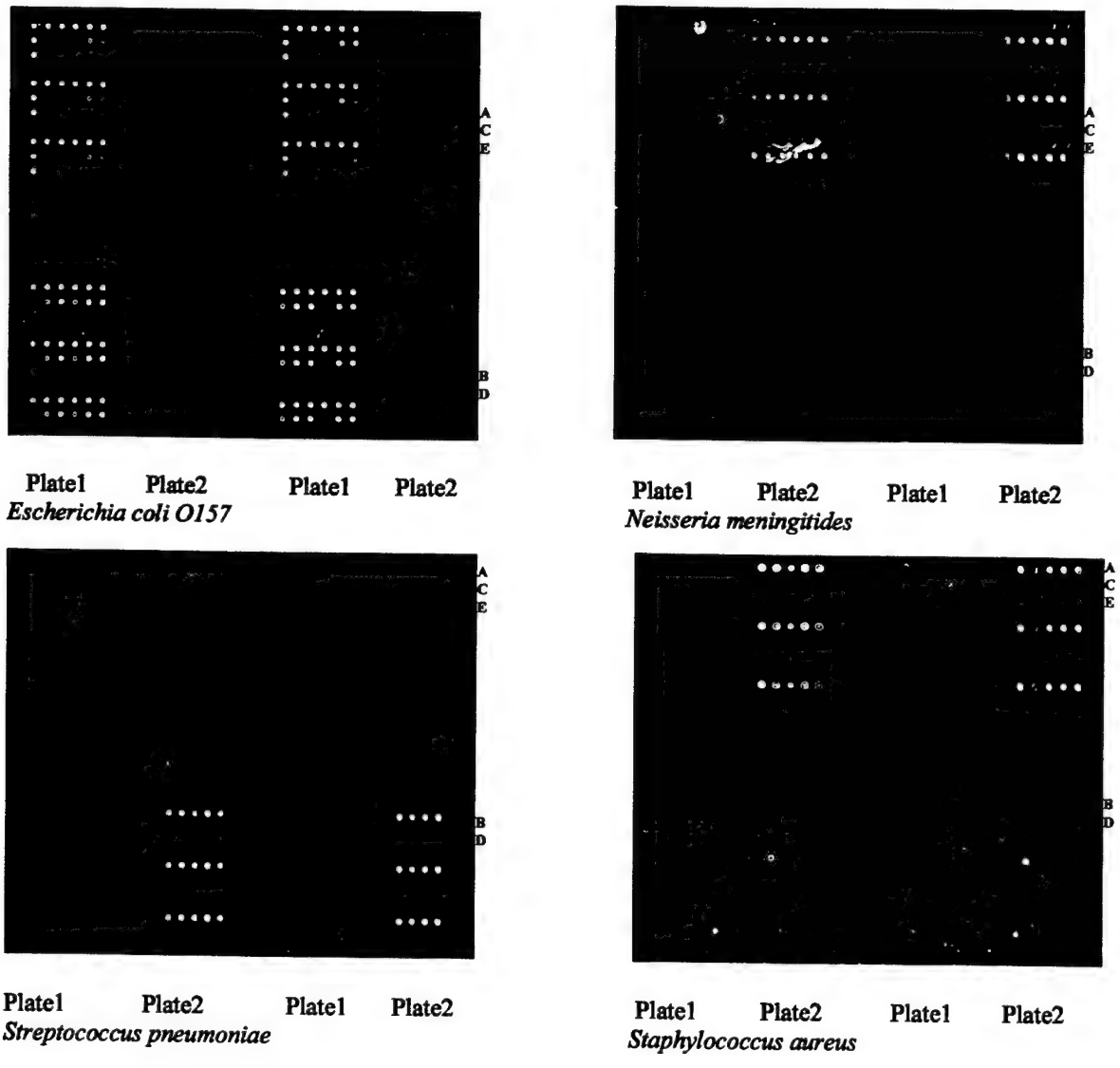

Figure 4. Discrimination of Four Different Pathogenic Bacteria

4. CONCLUSION

In summary, the selected genes are appropriate targets for accurately distinguishing pathogenic *E. coli* O157:H7, *S. aureus*, *S. pneumoniae*, and *N. meningitides*. Since the microarray can hybridize multiple genes simultaneously, the detection accuracy is significantly enhanced in comparison to traditional methods. In addition, a diversity of strains including not only wild-type, but also genetically modified variants, can be distinguished. This approach has great potential to facilitate high throughput, highly specific identification of bacterial and viral pathogens.

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